

Effect of Fish Oil Supplementation on the n-3 Fatty Acid Content of Red Blood Cell Membranes in Preterm Infants¹

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ABSTRACT. Very low birth weight infants demonstrate significant reductions in red blood cell membrane docosahexaenoic acid (DHA, 22:6n-3) following delivery unless fed human milk. The purpose of the present study was to determine if a dietary source of DHA (MaxEPA, R. P. Scherer Corporation, Troy, MI) could prevent the decline in red blood cell phospholipid DHA in very low birth weight infants whose enteral feeding consisted of a preterm formula without DHA. Longitudinal data were obtained on membrane phospholipid DHA in both unsupplemented and MaxEPA-supplemented infants by a combination of thin-layer and gas chromatography. These infants ($n = 39$) ranged in age from 10 to 53 days at enrollment (0 time). At enrollment, phospholipid DHA and arachidonic acid (20:4n-6) were inversely correlated with age in days. During the study, mean red blood cell phospholipid DHA declined without supplementary DHA as determined by biweekly measurement, but infants supplemented with MaxEPA maintained the same weight percent of phospholipid (phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine) DHA as at enrollment. The pattern of red blood cell phospholipid fatty acids in supplemented infants was similar to that reported for preterm infants fed human milk. (*Pediatr Res* 21: 507-510, 1987)

Abbreviations

DHA, 22:6n-3, docosahexaenoic acid
EPA, 20:5n-3, eicosapentaenoic acid
PE, phosphatidylethanolamine
PC, phosphatidylcholine
PS, phosphatidylserine
BHT, butylated hydroxytoluene

DHA (22:6n-3) normally comprises 30% or more of ethanolamine phosphoglyceride fatty acids in brain gray matter (1, 2)

Received August 1, 1986; accepted December 22, 1986.

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Supported in part by a grant-in-aid from Ross Laboratories, Columbus, OH.

¹ This study appeared in abstract form in *Pediatric Research* (26) and was presented at the 1986 meeting of the AAP/SPR in Washington, DC.

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and retinal membranes (3). Dietary manipulations which limit accumulation of DHA to 25-50% of controls in these membranes were associated with a 33% reduction in discrimination learning in rats (4), significant reductions in electroretinogram a-wave amplitudes (5), and a 25% loss of visual acuity in the rhesus monkey (5, 6). Furthermore, changes in electroretinograms observed after early deprivation persist following subsequent biochemical normalization of retinal membrane DHA in both species (5, 7). Accumulation of DHA in the central nervous system begins at about the 26th wk of gestation in the human fetus (8) with the weight percent of phospholipid DHA approaching adult levels by term (9). Studies in developing rats suggest that differences in red blood cell phospholipid DHA are indicative of qualitative differences in accumulation in the central nervous system.

Studies done in very low birth weight preterm infants showed that red blood cell PE DHA was high at birth, but the weight percent declined in the period before infants were nourished completely by orogastric feedings (1). Subsequently, an additional decline in PE DHA occurred in infants fed formula, whereas DHA increased in infants fed mother's milk (1).

Infant formula contains linolenic acid (18:3n-3) a precursor of DHA. Linoleic (18:2n-6) and linolenic acid are not interconvertible and compete for elongation and desaturation by the same enzyme system. Thus conversion of 20:3n-6 to 20:4n-6 and 20:4n-3 to 20:5n-3 is catalyzed by Δ^5 -desaturase and the elongation products of 20:4n-6 and 20:5n-3 (22:4n-6 and 22:5n-3, respectively) are converted to 22:5n-6 and 22:6n-3 by Δ^4 -desaturase.

Humans appear to have low Δ^4 -desaturase activity as indicated by the following observations: 1) diets enriched in 18:3n-3 increase phospholipid 20:5n-3 the Δ^5 -desaturase product, but not 22:6n-3, its Δ^4 -desaturase product (10); 2) red blood cell membrane phospholipid 22:5n-3 increases following birth (1, 11) while 22:6n-3 decreases in the absence of dietary 22:6n-3 (1); and 3) the decline in membrane 22:6n-3 in formula-fed infants is not accompanied by reciprocal increases in membrane 22:5n-6 (the Δ^4 -desaturase product of the linoleic acids series) (1, 11) in contrast to rats and monkeys with reduced membrane 22:6n-3 (4-7, 12).

In this study, red blood cell phospholipid DHA was used as an indicator of DHA status in preterm infants. Infants born at less than 1500 g were enrolled and followed biweekly with measurements of red blood cell phospholipid DHA. Enrollment occurred after the infants ceased to require respiratory support and were fed at least 60 kcal/kg/day from preterm formulas with or without a daily fish oil supplement (MaxEPA). MaxEPA is rich in both eicosapentaenoic (20:5n-3) (18-20%) and docosahexaenoic (12-14%) acids and was chosen because it did not contain potentially toxic amounts of vitamins A and D.

METHODS

Study population. Human infants born at less than 1500 g (range 600 to 1440) were eligible for this study. These infants were free of major congenital malformations and had no major disease process; e.g. bronchopulmonary dysplasia. Prior to entering the study, most infants required respiratory support for a short interval with either continuous positive airway pressure or intubation and mechanical ventilation for respiratory distress. Red blood cells were administered as necessary to maintain a hematocrit of 40% in infants until they reached 1500 g. The infants were cared for in incubators until weaning to a crib at approximately 1750 g and discharged at approximately 1800 g.

Experimental design. Infants became eligible for the study when they were receiving at least 60 kcal/kg/day of a formula designed for preterm infants without intravenous support. During the study most infants received either Similac Special Care (Ross Laboratories, Columbus, OH) or Enfamil Premature (Mead Johnson, Evansville, IN). Four infants were fed SMA "Premie" (Wyeth Laboratories, Philadelphia, PA). None of these formulas contained detectable 20 and 22 carbon polyunsaturated fatty acids.

A total of 61 infants were recruited into the protocol between May 1985 and January 1986 with informed consent obtained from a parent as approved by the Institutional Review Board of the University of Mississippi Medical Center. These infants were randomly assigned to receive preterm formula only ($n = 31$) or preterm formula with the fish oil supplement (MaxEPA, R. P. Scherer, Troy, MI, 750 mg/kg/day, $n = 30$) once daily by orogastric tube or in a nipple followed immediately by formula. A total of 106 infants less than 1300 g were admitted and survived during the 8-month study period. These infants constituted the pool from which all but three infants weighing between 1340 and 1440 g at birth were drawn. Selection was biased toward infants weighing less than 1200 g at the time they met the criteria for the study since larger infants were likely to exceed discharge weight before 4 wk of study.

Removal from the study was mandatory if feeding intolerance resulted in cessation of enteral feeding for more than 24 h. One supplemented infant was dropped from the study after 3 wk for this reason. A second infant recruited into the supplemented group became intolerant to orogastric feedings before supplementation began. Neither infant developed necrotizing enterocolitis. In addition, 11 infants from the unsupplemented group and eight infants from the supplemented group were lost by transfer to a level II nursery before completion of 4 wk of study. One infant from the unsupplemented group was excluded in the analysis because two of his three blood samples were oxidized. We planned to follow approximately 20 infants in each group for 4 wk. Assignment was randomized without regard for infants lost to the study as we anticipated losses would also be random. The infants in the subgroup studied for 6 wk were smaller and thus remained in the nursery longer before reaching discharge weight.

Data were available for a total of 19 unsupplemented and 20 supplemented infants followed for at least 4 wk. Unsupplemented compared to MaxEPA-supplemented infants had the following mean (\pm SD) birth weights (963 ± 209 and 944 ± 191 g), ages at entry into the study (28 ± 14 and 26 ± 10 days), and weight gain during 4 wk of study (547 ± 127 and 500 ± 98 g). None of these differences was significant. A subgroup of these infants was followed for an additional 2 wk: eight unsupplemented and 11 supplemented. The birth weights for these groups (mean \pm SD) were 867 ± 211 and 849 ± 146 g, respectively. Because of their lower birth weight they remained in the nursery longer before reaching discharge weight and a blood sample was obtained after 6 wk of study. Their ages at entry were 29 ± 18 and 27 ± 11 days, not different from the group as a whole.

Blood samples. Blood (1 ml) was removed from a small arm vein and added to EDTA to prevent coagulation. Blood samples

were obtained at enrollment (time 0) and at 2 and 4 wk. An additional blood sample was obtained at 6 wk from approximately one-half of the study infants. Blood was placed on ice and transported to the lab immediately where plasma was removed after centrifugation at 5° C. The erythrocytes were washed three times with 0.15 M NaCl, 1 mM EDTA, and resuspended in saline-EDTA. Both plasma and red blood cells were stored until analysis at -20° C in small Teflon-lined glass vials in a nitrogen atmosphere. Samples were stored under these conditions for less than 2 wk.

Preparation and analysis of phospholipid fatty acids. Red blood cell lipids were extracted according to the procedure of Dodge and Phillips (13) using chloroform and methanol. Methanol contained 50 mg/liter BHT as an antioxidant. The lipid extract was washed with 0.15 M KCl according to Folch *et al.* (14) and the organic solvent phase vaporized under nitrogen.

Phospholipid classes (PE, PC, PS) were separated on 10 \times 10 cm Silica gel G plates (Analtech, Newark, DE) with the solvent system of Zail and Pickering (15). PE, PC, PS, and sphingomyelin were identified by comparison with migration of standards and standard mixtures of these phospholipids (Supelco, Inc., Bellefonte, PA). Small bands were cut from either side of the plate, sprayed with 50% H₂SO₄ and charred to locate the individual phospholipids and confirm complete separation of each. The phospholipid bands from the uncharred portion of the plate were scraped immediately and methylated with boron trifluoride-methanol (Supelco, Inc.) in the absence of air according to Morrison and Smith (16). The methyl esters were stored at -20° C until analysis by gas chromatography.

The fatty acid methyl esters were separated and quantitated with a Shimadzu Mini 2 equipped with a Chromatopak CR1B. A 30 m long by 0.25 mm diameter open tubule column with a 0.2 μ m stationary phase of SP-2330 was used for separation (Supelco, Inc.). Carrier gas was helium. Column temperature was 210° C for 18 min advancing at 2° C/min for 8 min. The injector/detector temperature was 240° C. Identification of individual fatty acids was accomplished as detailed previously (11).

Statistical analysis. The analysis of the data consisted first of *t* test comparisons for each fatty acid. Two measures were compared: value at baseline and change over 4 wk (4-wk value - baseline). For all these *t* tests, the assumption of equal group variances was tested and when found to be violated, an adjusted *t* test was performed using the Satterthwaite (17) approximation. Two fatty acids (20:5n-3 and 22:6n-3) were selected for more complex statistical analysis (Tables 2 and 3). This analysis consisted of a multivariate analysis of variance of the complete 6-wk data (four measurements). Thus, only individuals with complete data for each fatty acid were used in the analysis. This reduced the sample size considerably and for this reason an identical analysis was done using the 4-wk data. The multivariate approach was chosen over the corresponding repeated measures analysis because it does not require the assumption of a particular covariance structure inherent in the latter. For each analysis, the following effects were tested: overall group effect (unsupplemented *versus* MaxEPA-supplemented), time effect (overall change over time), and group \times time interaction (different rates of change for each group). The time effect was subdivided into linear and deviation from linear components. Thus the time linear \times group interaction tests the equality of slopes of the time response in the two groups. Details of this type of analysis can be found in Grizzle and Allen (18). All statistical analyses were performed using the SAS data analysis package (19).

RESULTS

Phospholipid fatty acids at enrollment. At the time of enrollment, the 39 infants in this study were between 10 and 53 days of age. PE DHA varied from 3.6 to 9.3% of total fatty acids, and there was a highly significant inverse correlation between age

and red blood cell PE DHA (Table 1). PE arachidonate (20:4n-6) varied from 19.4 to 30.3% of total fatty acids, and also correlated inversely with age. PC DHA and arachidonate ranged from 0.1 to 1.8% and from 5.0 to 16.3%, respectively. They too were inversely correlated with age (Table 1).

Mean baseline values for DHA and arachidonate in PE, PC, and PS of unsupplemented and MaxEPA-supplemented infants did not differ; neither did the mean values for all other fatty acids in these phospholipids differ at enrollment.

The data were also analyzed for the sum of 20 to 22 carbon polyunsaturated fatty acids (20:2n-6 + 20:3n-6 + 20:4n-6 + 20:5n-3 + 22:4n-6 + 22:5n-6 + 22:5n-3 + 22:6n-3), the sum of n-3 fatty acids with more than 18 carbons (20:5n-3 + 22:5n-3 + 22:6n-3), the sum of n-6 fatty acids with more than 18 carbons (20:2n-6 + 20:3n-6 + 20:4n-6 + 22:4n-6 + 22:5n-6), the ratio of 20:4n-6/20:5n-3 and the ratio of Δ⁴-desaturase products (22:5n-6 + 22:6n-3) to substrates (20:4n-6 + 22:4n-6 + 20:5n-3 + 22:5n-3). The degree of unsaturation of the membrane was determined by the following equation: Σ[(% each fatty acid) (number of double bonds/fatty acid)]. Baseline values did not differ for unsupplemented compared to supplemented infants.

Phospholipid fatty acids during the study period. Individual fatty acids and the combinations listed above were analyzed for change with 4 wk of treatment. Those which were significant are presented below. The additional data may be obtained from S.E.C. In each phospholipid class analyzed, unsupplemented and supplemented infants differed in the change in DHA during 4 wk of study (PE, *p* < 0.006; PC, *p* < 0.001; PS, *p* < 0.01), Tables 2 and 3 include the biweekly mean weight percent of DHA in PE and PC of red blood cells during the course of the study. The DHA of PE and PC declined between 0 and 2 wk, and between 2 and 4 wk, with a further decline between 4 and 6 wk in the subgroup studied longer (Table 2). MaxEPA-supplemented infants maintained the same weight percent of DHA in PE, while

Table 1. Relationship between time after preterm delivery and red blood cell phospholipid docosahexaenoate (22:6n-3) and arachidonate (20:4n-6) (*n* = 39)

Phospholipid	Fatty acid	Correlation* (<i>r</i>)	Significance (<i>p</i>)
PE	20:4n-6	-0.43	<0.006
	22:6n-3	-0.47	<0.002
PC	20:4n-6	-0.50	<0.001
	22:6n-3	-0.43	<0.005

* Age (days) at enrollment correlated with each fatty acid.

Table 2. Red blood cell PE and PC docosahexaenoic acid (22:6n-3) (weight percent, mean ± SEM)*, †

Time	PE		PC	
	Unsupple-mented	Supplemented	Unsupple-mented	Supplemented
A				
	(19)	(20)	(19)	(20)
0	6.2 ± 0.4	6.2 ± 0.3	0.89 ± 0.13	1.01 ± 0.09
2 wk	5.1 ± 0.3	5.9 ± 0.2	0.69 ± 0.06	1.05 ± 0.06
4 wk	4.4 ± 0.2	5.8 ± 0.2	0.60 ± 0.05	1.27 ± 0.08
B				
	(8)	(11)	(8)	(11)
0	6.0 ± 0.7	5.9 ± 0.4	1.01 ± 0.22	0.96 ± 0.12
2 wk	5.2 ± 0.6	5.9 ± 0.4	0.69 ± 0.11	0.95 ± 0.08
4 wk	4.2 ± 0.4	5.6 ± 0.3	0.60 ± 0.10	1.28 ± 0.17
6 wk	3.7 ± 0.4	5.9 ± 0.3	0.57 ± 0.08	1.32 ± 0.20

* A: infants studied 4 or 6 wk; B: subset studied for 6 wk.

† PE, group-time interaction (linear): A, *p* < 0.004; B, *p* < 0.002. PC, group-time interaction: A, *p* < 0.001; B, *p* < 0.005. PE, group: A, *p* < 0.033. PC, group: A, *p* < 0.001; B, *p* < 0.02. PE, time (linear): A, *p* < 0.001.

Table 3. Red blood cell PE and PC eicosapentaenoic acid (20:5n-3) (weight percent, mean ± SEM)*, †

Time	PE		PC	
	Unsupple-mented	Supplemented	Unsupple-mented	Supplemented
A				
	(19)	(20)	(19)	(20)
0	0.27 ± 0.04	0.41 ± 0.08	0.12 ± 0.02	0.16 ± 0.04
2 wk	0.32 ± 0.04	0.68 ± 0.07	0.18 ± 0.07	0.48 ± 0.08
4 wk	0.41 ± 0.04	0.85 ± 0.08	0.13 ± 0.03	0.54 ± 0.07
B				
	(8)	(11)	(8)	(11)
0	0.20 ± 0.05	0.36 ± 0.07	0.10 ± 0.02	0.21 ± 0.07
2 wk	0.30 ± 0.06	0.74 ± 0.11	0.07 ± 0.03	0.47 ± 0.40
4 wk	0.26 ± 0.03	0.85 ± 0.14	0.11 ± 0.12	0.57 ± 0.38
6 wk	0.40 ± 0.05	1.02 ± 0.16	0.14 ± 0.05	0.57 ± 0.34

* A: infants studied 4 or 6 wk; B: subset studied for 6 wk.

† PE, group-time interaction (linear): A, *p* < 0.002; PC, group-time interaction (linear): A, *p* < 0.001. PE, group: A, *p* < 0.001. PC, group: A, *p* < 0.001. PE, time (linear): A, *p* < 0.002. PC, time (linear): A, *p* < 0.001.

the weight percent increased slightly in PC (Table 2). PS had a level of DHA very similar to that of PE at enrollment and after 4 wk in both unsupplemented and MaxEPA supplemented infants: 6.8 ± 0.4 with a mean loss of 1.9 ± 0.4% in unsupplemented and a decrease of 0.4 ± 0.4% in supplemented infants.

Biweekly values for PE and PC eicosapentaenoate are shown in Table 3. There was a significant time by treatment relationship for eicosapentaenoate in the group as a whole with significant increases in PE and PC of supplemented infants (Table 3).

When the data were analyzed for the change from baseline during 4 wk of study, 20:5n-3, 22:6n-3, and the Σn-3 >18C consistently showed highly significant treatment differences with values in MaxEPA-supplemented infants reflecting inclusion of these fatty acids in the diet. Phospholipid 22:5n-3 accounted for 1.6 (PE), 0.2 (PC), and 1.3 (PS) % of total fatty acids at baseline. While 22:5n-3 increased in all phospholipid classes, treatment effects were significant only for PC and PS (*p* < 0.02) reflecting intakes of small amounts of 22:5n-3 by MaxEPA-supplemented infants.

The change in the ratio of 20:4n-6/20:5n-3 was significant only in PC (112.4 ± 15.5 with a mean increase of 46.5 ± 30.4 in untreated and 90.9 ± 11.7 with a mean decrease of 71.7 ± 12.9 in treated infants, *p* < 0.001).

In addition PE, PC, and PS had ratios of Δ⁴-desaturase products/substrates after 4 wk identical to baseline, while this ratio declined in PE (*p* < 0.001) and PC (*p* < 0.04) of infants without supplementation.

Neither the sum of n-6 metabolites with 20 and 22 carbons nor the degree of unsaturation of membrane phospholipids were affected by the fish oil treatment. Neither were significant differences noted for 22:4n-6 and 22:5n-6 with time, treatment, or time x treatment. This was surprising since the Δ⁴-desaturase product of the linoleic (n-6) family, 22:5n-6, increased when DHA was reduced in membranes of rats and monkeys fed diets with very high ratios of linoleic (18:2n-6) to linolenic (18:3n-3) acid (6, 12, 20).

DISCUSSION

Results of this study indicate that long-term maintenance of DHA levels in red blood cell phospholipids may be achieved by fish oil (MaxEPA) supplementation. After 6 wk of supplementation, the weight percent of DHA in membrane phospholipids was similar to that reported previously (1) for infants fed preterm human milk for a similar period of time. MaxEPA required to

prevent a decline in membrane DHA (750 mg/kg/day) provided approximately six times as much DHA as would have been received by infants fed preterm human milk, but this dose was employed only after 250 mg/kg/day of MaxEPA in a preliminary trial failed to prevent the linear decline in membrane DHA. A follow-up study confirmed that digestion and absorption of fish oil was quite limited in these infants receiving a bolus of MaxEPA daily at the time of a single formula feeding, but indicated that good digestion of marine oil triglycerides may be achieved in preterm infants (21) despite the expected low levels of pancreatic lipase, limited ability of pancreatic lipase to hydrolyze marine oil triglycerides (22), or a combination of these factors.

It is unlikely that the membrane DHA observed at the conclusion of the feeding portion of this study reflects the lowest membrane DHA reached in these infants. The mean weight percent of PE DHA in unsupplemented infants fell linearly in the periods between 0 and 2, 2 and 4, and 4 and 6 wk. After 6 wk of study the mean PE DHA at 6 wk (3.7%, $n = 8$) was still higher than observed in unsupplemented infants returning to follow-up clinic 10 and 12 wk after enrollment (2.6%, $n = 4$).

Data from this study confirm our earlier reports that dietary 22:6n-3 is more important than 18:3n-3 in contributing to membrane DHA in human infants (1, 11) and that declines in 22:6n-3 are not accompanied by increases in 22:5n-6 in membrane phospholipids. During the study period, the ratio of phospholipid fatty acid products of Δ^4 -desaturation to all products of Δ^5 -desaturation was consistently maintained only in the group receiving the dietary Δ^4 -desaturase product DHA; i.e. the group supplemented with MaxEPA. These data are consistent with limited Δ^4 -desaturation in these infants.

Longitudinal data from this study confirm suggestive data from an earlier report (1) that the DHA content of red blood cell phospholipids in preterm infants decreases following birth unless a source of dietary DHA is administered. Poor DHA accumulation in brain and retinal membrane phospholipids during development results in functional impairment in the rat and rhesus monkey (4, 7), and one report in the rhesus monkey indicated that the period of accumulation may be critical (7). Although similar evidence of functional impairment related to poor DHA accumulation has not been demonstrated in very low birth weight infants, those with normal intelligence at school age nevertheless have significantly poorer school performance compared to their peers born at term. In at least two recent reports, poor school performance was associated with some degree of impaired visual-motor function (23, 24).

A test for measuring visual acuity in infants is available (25). It would allow a test of the hypotheses that visual acuity is impaired in very low birth weight infants compared to infants born at term unless the decline in membrane DHA is prevented by addition of small amounts of dietary DHA. Such trials would depend on maintenance of membrane DHA levels in a range similar to cord blood until an adjusted age of term followed by a measurement of visual function. Before these trials can be undertaken, a source of DHA must be incorporated into infant formula which will provide long-term maintenance of membrane phospholipid DHA in a physiological range without risk to the infant. This study provides evidence that maintenance of membrane DHA can be achieved by dietary supplementation with a source of DHA but does not offer a practical means of maintaining DHA following hospitalization. This problem is currently being studied.

Acknowledgment. MaxEPA was supplied by R. P. Scherer Corporation, Troy, MI.

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